Chapter 2

Alternative Cell Sources to Adult Hepatocytes for Hepatic Cell Therapy

Eugenia Pareja, María José Gómez-Lechón, and Laia Tolosa

Abstract

Adult hepatocyte transplantation is limited by scarce availability of suitable donor liver tissue for hepatocyte isolation. New cell-based therapies are being developed to supplement whole-organ liver transplantation, to reduce the waiting-list mortality rate, and to obtain more sustained and significant metabolic correction. Fetal livers and unsuitable neonatal livers for organ transplantation have been proposed as potential useful sources of hepatic cells for cell therapy. However, the major challenge is to use alternative cell sources for transplantation that can be derived from reproducible methods. Different types of stem cells with hepatic differentiation potential are eligible for generating large numbers of functional hepatocytes for liver cell therapy to treat degenerative disorders, inborn hepatic metabolic diseases, and organ failure. Clinical trials are designed to fully establish the safety profile of such therapies and to define target patient groups and standardized protocols.

Key words Clinical trials, Fetal hepatocytes, Induced pluripotent stem cells, Neonatal hepatocytes, Pluripotent stem cells, Inborn metabolic errors, Liver transplantation, Hepatocyte transplantation

1 Introduction

Cell-based therapies have been a particularly active research area in recent years, whose objective has been to restore lost organ function. Hepatocyte transplantation (HT) has been considered worldwide a promising alternative to liver transplantation (LT) for a variety of indications, including acute liver failure (ALF) and metabolic liver diseases [1–8]. Cell transplantation offers a number of potential advantages compared with LT. The procedure is considerably less invasive, with less risk of morbidity and mortality. Unlike whole organs, cells can be cryopreserved and stored until needed. So cells are available immediately for both programmed treatment, which can be performed repeatedly for liver-based metabolic disorders, and emergency use in patients with ALF when an organ is not available. However at present, HT is limited by scarce availability of suitable donor liver tissue for hepatocyte isolation. Other major
concerns include the detrimental effects of cryopreservation on the viability and metabolic function of adult hepatocytes [6, 9–11] and, despite encouraging results, long-term sustained therapeutic benefits of adult HT are generally lacking due to allograft rejection. Therefore, the ability to reproducibly generate a well-characterized source of engraftable and functional liver cells has remained a challenge.

New cell-based therapies are being developed to supplement whole-organ liver transplantation, to reduce the waiting-list mortality rate, and to obtain more sustained and significant metabolic correction [4, 12]. Fetal livers [7, 13–16] or neonatal livers [17–19] unsuitable for organ transplantation have been proposed as potential useful sources of hepatic cells for cell therapy. Fetal and neonatal hepatocytes are also less vulnerable to cryopreservation than adult hepatocytes [19, 20]. Notwithstanding, the major challenge for this field is to identify alternative reliable cell sources for transplantation, the equivalent to hepatocytes, which can be derived from reproducible methods. Replacing hepatocytes with hepatocyte-like cells (HLCs) generated from stem cells is an alternative strategy to overcome shortage of hepatocytes. Stem cells that have the potential to be expanded, maintained, and differentiated in cell cultures are promising to help improve the efficacy of hepatic cell-based therapy for treating and repairing damaged tissue in the future [4, 21–23].

Different types of stem cells with hepatic differentiation potential are eligible for generating large numbers of functional hepatocytes for liver cell therapy. These include pluripotent stem cells (PSCs), comprising embryonic stem cells (ESCs), induced pluripotent stem cells (iPSCs), and adult stem cells, such as hepatic progenitor cells (HPCs), amnion-derived stem cells (ASCs), and mesenchymal stem cells (MSCs). Human PSCs-derived hepatocytes are emerging as cell-based systems that may potentially provide a stable source of hepatocytes for multiple applications, including cell therapy. Different protocols have been developed to isolate ESCs and to induce them to form HLCs by mimicking the developmental pathway of the liver during embryogenesis [24, 25]. The recently described induced pluripotent stem cells (iPSCs) might circumvent ethical concerns about embryonic and fetal liver stem cells. iPSC-derived hepatocytes have been shown to be most promising in terms of acquiring a primary tissue-like phenotype and unlimited availability [4, 25, 26]. Although current differentiation protocols of iPSCs to hepatic cells need to be optimized, they can provide a limitless supply of hepatocytes, which implies the additional benefit of being able to provide patient-specific hepatocytes.

In vitro expansion makes it possible to break away from dependency on organ donation and is also compatible with large-scale pharmaceutical production, which provides a real prospect of
bringing hepatic cell-based therapy to any patient in need in any metabolic center. Pharmaceutical development is also the guarantee to conduct proper clinical trials to evaluate both safety and efficacy. Stem or progenitor cells, produced in vitro under good manufacturing practice conditions, are classed as medicinal products in Europe. Ideally, these cell lines would be highly viable preparations with robust hepatic function and engraftment capacity, and well characterized.

In summary, cell-based therapies, particularly those based on stem cells or more differentiated progenitor cells, are promising tools at the service of regenerative medicine to treat degenerative disorders, inborn hepatic metabolic diseases, and organ failure [4, 5, 12, 21]. Nonetheless, critical aspects need to be further addressed, including the long-term safety, tolerability and efficacy of these stem cell-based treatments, as well as their tumorigenic potential. Consequently, it is paramount to conduct larger well-designed clinical trials to fully establish the safety profile of such therapies and to define target patient groups with efficacy assessed by standardized protocols.

2 Clinical Hepatic Cell Therapy Applications

LT is currently the treatment of choice for end-stage liver diseases and life-threatening liver-based metabolic disorders. Inborn errors of metabolism affect around 1/900 live births. This pathology often represents rare conditions characterized by accumulation of metabolic intermediates in organs and physiological fluid. For some metabolic disorders, the risks associated with LT are not justified, and HT could be a less invasive option to improve these patients’ long-term outcome [1].

Theoretically, all liver disorders that are attributable to a single gene defect have the potential to benefit from HT. However, only a small number of disorders have been targeted to date. Management of patients with metabolic diseases is very complex and includes orphan medications, specific diets, and special education. Besides life-threatening conditions, one main concern is that long-term intellectual prognosis may be impaired. The quality of life of patients and their family is often poor.

The indication for HT is currently based on disease severity or quality of life, and the goal is to avoid or postpone LT, be it due to inborn genetic metabolism errors or ALF [1, 6, 27].

The most encouraging outcomes of HT have been reported in patients with metabolic liver-based disorders [7, 28]. HT is, therefore, a promising alternative, especially in diseases with a nearly intact liver, but with systemic organ damage. Clinical studies into disorders, such as glycogen storage disorders and urea cycle defects, have already highlighted the corrective capacity of HT to improve
clinical outcomes, and it is reasonable to assume that additional advances with HT can be expected [29–32]. The obtained results are encouraging, but the cell transplantation protocols used in different centers vary. Therefore, outcomes are difficult to compare. The lower surgical risk and fewer consequences of graft loss associated with HT, as opposed to LT, could benefit patients with these diseases. Deciding the best timing for transplantation in these patients is challenging. The general rule is to try to postpone surgery and exposing children to immunosuppressive drugs as much as possible. The first attempt to treat inherited metabolic liver disease with HT was made in the ex vivo gene therapy for familial hypercholesterolemia. A trial that involves four patients has demonstrated a slight reduction in plasma cholesterol levels and the persistence of transplanted cells. Although the procedure did not effectively lower LDL levels, the trial established the feasibility of HT and the longevity of transplanted cells [33]. Table 1 summarizes the commonest liver metabolic disorders in which HT has been clinically applied. The results are encouraging, although the cell transplantation protocols used in different centers vary. So outcomes are difficult to compare.

HT has also been envisaged as a useful therapeutic approach for bridging patients to LT, and is indicated for providing metabolic support during ALF and acute on chronic liver failure in which the only hope for survival for most patients is either LT, or facilitating liver regeneration in cases of acute or fulminant hepatic failure or a major resection for metastatic disease [1, 5, 34]. In fact, the first HT clinical trials were reported by Mito and Kusano, who injected isolated human hepatocytes into the spleen of ten Japanese patients with liver cirrhosis or chronic hepatitis [35].

3 Alternative Cell Sources to Adult Hepatocytes

The worldwide shortage of donor livers for HT has prompted the search for alternative cell therapies for intractable liver diseases (Fig. 1). Current sources of liver tissue are mainly adult organs rejected for transplantation, normally of marginal quality, such as severe steatosis, prolonged cold ischemia time, and older donors. Despite improvements in hepatocyte isolation methods, it is well known that the mature hepatocytes obtained from these livers often show poor and insufficient functional quality and viability [4, 21, 36]. Therefore, other sources of human hepatic tissue for hepatocyte isolation have been explored. One interesting alternative to the adult liver is the use of fetal livers or neonatal livers for unsuitable organ transplantation as potential sources of good-performing hepatic cells. Although their use in cell transplantation has been poorly explored, they have several advantages compared to adult liver cells: availability, proliferative capacity,
### Table 1
Clinical indications of hepatocyte transplantation

<table>
<thead>
<tr>
<th>Inborn hepatic metabolic diseases</th>
<th>Clinical case</th>
<th>Follow up after HT</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Crigler–Najjar syndrome type 1</strong></td>
<td>10-year-old female. 7.5 × 10⁹ hepatocytes (5% liver mass)</td>
<td>Reduction of phototherapy, Reduction of bilirubin levels, Excretion of conjugated bilirubin. OLT, 4 years after HT</td>
<td>[136]</td>
</tr>
<tr>
<td>8-year-old female. 7.5 × 10⁹ hepatocytes</td>
<td>40% Reduction of bilirubin levels. OLT, 20 months after HT</td>
<td>[29]</td>
<td></td>
</tr>
<tr>
<td>9-year-old boy. 7.5 × 10⁹ hepatocytes (5% liver mass)</td>
<td>30% Reduction of bilirubin levels. Inadequate phototherapy → OLT 5 months after HT</td>
<td>[137]</td>
<td></td>
</tr>
<tr>
<td>18-month-old. 4.3 × 10⁹ hepatocytes</td>
<td>40% Reduction of bilirubin to 7 months. OLT at 8 months</td>
<td>[138]</td>
<td></td>
</tr>
<tr>
<td>3-year-old girl. 2.1 × 10⁹ hepatocytes</td>
<td>No clear benefit. OLT at 18 months</td>
<td>[138]</td>
<td></td>
</tr>
<tr>
<td>9-year-old female. 6.1 × 10⁹ hepatocytes (4% liver mass)</td>
<td>30% reduction of serum bilirubin. OLT 6 months after HT</td>
<td>[139]</td>
<td></td>
</tr>
<tr>
<td>1-year-old female. 2.6 × 10⁹ hepatocytes. (8.6% liver mass)</td>
<td>25% reduction of serum bilirubin. OLT 4 months after HT</td>
<td>[139]</td>
<td></td>
</tr>
<tr>
<td>7-year-old female. 1.4 × 10⁹ hepatocytes (&lt;1% liver mass)</td>
<td>40% reduction of serum bilirubin levels after HT and 50% reduction in phototherapy. OLT after 11 months</td>
<td>[140]</td>
<td></td>
</tr>
<tr>
<td>11-year-old male. 7.2 × 10⁹ hepatocytes</td>
<td>20% reduction of bilirubin levels. OLT</td>
<td>[141]</td>
<td></td>
</tr>
<tr>
<td>7-month-old female. 6.7 × 10⁹ hepatocytes. (17% liver mass)</td>
<td>Decrease of bilirubin levels (25 mg/day to 14) 50% reduction of phototherapy</td>
<td>[31]</td>
<td></td>
</tr>
</tbody>
</table>

Urea cycle defects
The table below continues from the previous page. It details the clinical cases and outcomes of individuals with inborn hepatic metabolic diseases.

<table>
<thead>
<tr>
<th>Inborn hepatic metabolic diseases</th>
<th>Clinical case</th>
<th>Follow up after HT</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Ornithine transcarbamylase (OTC)</strong></td>
<td>5-year-old boy. $1 \times 10^9$ hepatocytes</td>
<td>Ammonia levels normalized. Died from pneumonia 42 days after HT</td>
<td>[142]</td>
</tr>
<tr>
<td></td>
<td>10-month-old male. $4 \times 10^9$ hepatocytes (22% liver mass)</td>
<td>Normalization of ammonia and glutamine levels on normal protein diet. OLT at 6 months of age</td>
<td>[30]</td>
</tr>
<tr>
<td></td>
<td>1-day-old male. $1.74 \times 10^9$ hepatocytes (5% liver mass)</td>
<td>Decrease of ammonia levels and increase in serum urea. Auxiliary partial OLT performed at 7 months of age</td>
<td>[143]</td>
</tr>
<tr>
<td></td>
<td>14-month-old boy. $3.5 \times 10^9$ hepatocytes</td>
<td>Blood ammonia level decreased. Urea levels increased. OLT 6 months after HT</td>
<td>[144]</td>
</tr>
<tr>
<td></td>
<td>6-h-old male. $0.64 \times 10^9$ hepatocytes (4% liver mass)</td>
<td>No metabolic crises occurred in the neonatal period. Died at 4 months of age by norovirus infection</td>
<td>[17, 18]</td>
</tr>
<tr>
<td></td>
<td>9-days-old male. $0.56 \times 10^9$ hepatocytes (4% liver mass)</td>
<td>Blood ammonia level decreased. Protein intake increased. OLT</td>
<td>[17, 18]</td>
</tr>
<tr>
<td><strong>Carbamoylphosphate synthetase type 1 (CPS1) deficiency</strong></td>
<td>12-year-old girl. $0.87 \times 10^9$ hepatocytes (2% liver mass)</td>
<td>Blood ammonia level decreased. Urea levels increased. Died from septic shock 30 days after HT</td>
<td>[31]</td>
</tr>
<tr>
<td><strong>Citrullinemia</strong></td>
<td>2.5-month-old male. $1.37 \times 10^9$ hepatocytes</td>
<td>Normalization of ammonia. Urea production and protein intake increased. OLT</td>
<td>[17, 18]</td>
</tr>
<tr>
<td></td>
<td>3-year-old female. $1.46 \times 10^9$ hepatocytes (3% liver mass)</td>
<td>Normalization of ammonia. 40% increase in urea. Increased protein intake 10 months after HT. OLT</td>
<td>[17, 18]</td>
</tr>
<tr>
<td><strong>Argininosuccinatelyase (ASL) deficiency</strong></td>
<td>2-year-old-female. $3 \times 10^9$ hepatocytes</td>
<td>Decrease of citrulline and ammonia at 2 weeks to 6 months</td>
<td>[145]</td>
</tr>
<tr>
<td></td>
<td>3.5-year-old female. $3 \times 10^9$ hepatocytes (10% liver mass)</td>
<td>Decrease of ammonia levels. Metabolic control Psychomotor Catch-up. OLT after 18 months</td>
<td>[146]</td>
</tr>
<tr>
<td>Inborn hepatic metabolic diseases</td>
<td>Clinical case</td>
<td>Follow up after HT</td>
<td>Reference</td>
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<tr>
<td>Glycogen storage disease type I</td>
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<td><strong>Type I a</strong></td>
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<tr>
<td>47-year-old female.</td>
<td>Improved blood glucose control.</td>
<td>[147]</td>
<td></td>
</tr>
<tr>
<td>2 × 10⁹ hepatocytes (1% liver mass)</td>
<td>Decrease in blood lactate concentration.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6-year-old female.</td>
<td>Improved blood glucose, cholesterol, and triglycerides control.</td>
<td>[31]</td>
<td></td>
</tr>
<tr>
<td>2.35 × 10⁹ hepatocytes (17% liver mass)</td>
<td>Decrease of blood lactate concentration. No hypoglycemic episodes</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Type I b</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>18-year-old male.</td>
<td>Improved blood glucose control.</td>
<td>[148]</td>
<td></td>
</tr>
<tr>
<td>0.17 × 10⁹ hepatocytes (6% liver mass)</td>
<td>Decreased blood lactate concentration.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Refsum disease</td>
<td></td>
<td>40% decrease of pipecolic acid</td>
<td>[149]</td>
</tr>
<tr>
<td>4 year-old female.</td>
<td>18 months after HT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.1 × 10⁹ hepatocytes (5% liver mass)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phenylketonuria</td>
<td></td>
<td>70% reduction in phenylalanine level.</td>
<td>[150]</td>
</tr>
<tr>
<td>6-year-old male.</td>
<td>Half-life of phenylalanine decreased from 41.6 to 19.1 h</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.58 × 10⁹ hepatocytes</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tyrosinemia type 1</td>
<td></td>
<td>Improvement in clotting factors levels. Bilirubin levels decreased.</td>
<td>[31]</td>
</tr>
<tr>
<td>45-day-old male.</td>
<td>OLT at 4 months</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.66 × 10⁹ hepatocytes (3.5% liver mass)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Factor VII deficiency</td>
<td></td>
<td>70% decrease of their requirements for recombinant FVII (rFVIIa).</td>
<td>[138]</td>
</tr>
<tr>
<td>3-month-old male.</td>
<td>OLT at 7 months</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.1 × 10⁹ hepatocytes (4% liver mass)</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>3-year-old male.</td>
<td>70% decrease of requirements of recombinant FVII (rFVIIa).</td>
<td>[138]</td>
<td></td>
</tr>
<tr>
<td>2.2 × 10⁹ hepatocytes (3% liver mass)</td>
<td>OLT at 8 months</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3-month-old male.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3.6 × 10⁹ (unpub)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Primary hyperoxaluria</td>
<td></td>
<td>Plasma oxalate continuously decreased. OLT at 12 months</td>
<td>[151]</td>
</tr>
<tr>
<td>1.3-year-old-female.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.1 × 10⁹ hepatocytes</td>
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</tbody>
</table>
good adaptation and integration capacity into the host liver, and plasticity for fetal hepatic cells. PSCs may offer many advantages as an alternative source of cells for cell therapy. They are readily available and may be effectively expanded in vitro or in vivo, and differentiated into hepatocytes. ESCs cells have also been suggested to possibly be more resistant to cryopreservation and less immunogenic. The equivalent to ESCs in gene expression are iPSCs, which can be generated from human skin by the retroviral transduction of transcription factors [37] to generate suitable populations of human hepatocytes for personalized hepatic cell therapy. Ex vivo gene-corrected patient-specific iPSCs lines can also be used for autologous transplantation as a therapeutic option [4].

The challenges associated with the clinical use of stem cells are considerable. For example, perfecting methods to the scale-up production of hepatocytes from stem cells is a difficult, but necessary, step because vast numbers of cells are required for transplants to be effective. Before basic stem cell research can be translated into clinical practice with patients, it must be first rigorously tested and validated in preclinical studies.

### 3.1 Fetal Hepatocytes

Fetal liver [7, 13–15, 38] has been considered a potential suitable alternative cell source for hepatic cell therapy. Traditional hepatocyte isolation techniques for low-gestational-age fetal livers are based on the mechanical disruption of tissue into small fragments, and then incubation with collagenase for the digestion of
connective tissue, which yields only a fraction of the number of available cells. Therefore, the clinical application of fetal hepatocytes from 12-week gestation livers is restricted given the difficulty of obtaining large numbers of fetal liver cells. For example, to transplant a quantity of cells that corresponds to 2% of the liver mass in a newborn, around 30 of these fetal livers are needed [8]. However, a five-step method for donated tissue from gestation weeks 18 to 22 has been reported, in which a portal vein perfusion technique resulted in wide viabilities [38]. Briefly, the liver was perfused in situ for 10 min at 37 °C with a buffered solution that contained chelating agent ethylenediamine tetraacetic acid (EGTA) to loosen the desmosomal cell–cell junctions. Thereafter the liver was perfused for 10 min with the solution without EGTA to prepare tissue for digestion by collagenase perfusion with a calcium-containing solution for 7–10 min until the organ became soft and tissue started to disintegrate. Any undigested tissue was removed by filtration and the cell suspension was washed 2–3 times by low-speed centrifugation. The procedure yielded between 3.8 and 13 × 10⁷ cells per gram of liver tissue, and an average cell viability of 78%, as determined by the trypan blue exclusion method [38].

Fig. 1 Cell sources for hepatic cell therapy. Hepatocytes can be obtained from fetal livers, or unused neonatal or adult livers for OLT. Embryonic stem cells (hESC) are available and can be expanded and differentiated into hepatocytes. Induced pluripotent stem cells (iPSCs), generated from human skin cells, can be differentiated into human hepatocytes for personalized hepatic cell therapy. Other stem cell sources can also be used.
Regarding the hepatic markers of cells, the human fetal liver obtained during the first 12 weeks of gestation mainly exhibits cells that express hematopoietic [39, 40] and endothelial cell markers [41], while the majority of cells express hepatic markers appear early in the second trimester [42]. Nonetheless, the resulting cell preparations from the fetal liver late in the second trimester phenotypically show, in addition to hepatic progenitors, cells that express typical mesenchymal stem cells and hematopoietic stem cells markers [38, 43]. The percentage of fetal liver cells that express proliferation markers is 45 times higher than the percentage of adult hepatocytes that express these markers [38]. Indeed, a larger number of cells positive for the epithelial cell adhesion molecule (EpCAM), a transmembrane glycoprotein that is expressed in hepatic progenitors, have been described in fetal livers [43]. So even though human fetal liver cells are not likely to be routinely used for clinical liver cell repopulation, future trends will focus on the potential of fetal liver stem/progenitor cells to repopulate the liver [44].

Fetal hepatocytes are immature and hepatocytic traits depend on the gestational age. It has been reported that 20–22-week gestation fetal hepatocytes perform liver-specific functions at levels comparable to those of their adult counterpart, while the values of liver-specific functions in cells from 16 to 18 gestation weeks are very low [20]. Based on current knowledge, drug-metabolizing enzymes are expressed at negligible or low levels in fetal liver [45, 46]. Moreover, the fetal expression patterns of CYP450 enzymes considerably vary. Although substantial developmental changes in the hepatic expression in both CYP2C9 and CYP2E1 occur, the expressions of CYP2C19, CYP3A5, and CYP3A7 remain relatively constant in the fetal liver [45, 46], with CYP3A7 accounting for up to 50% of total fetal hepatic CYP450 content [47]. Regarding conjugating enzymes, the expression of UDP-glucuronosyltransferase 1A1 (UGT1A1), the major enzyme responsible for bilirubin glucuronidation, is absent or very weak in the fetal liver [46, 48]. However, UGT1A6, UGT1A9, and UGT2B7 isozymes have already been seen to be expressed in the fetal liver, but develop slowly during the postnatal period [49, 50]. Some authors have proposed the hepatic maturation of fetal hepatocytes in primary culture to generate fully differentiated hepatocytes for clinical use [51, 52]. Fetal hepatocytes, with a high regenerative capacity, have shown a mature hepatic phenotype, established by gene expression profiling, and functional integration within the first few weeks after transplantation into the host liver [13, 14].

Clinical studies that used fetal hepatocytes have suggested that they are a potentially useful source of cells for clinical therapy. In agreement with this, a 1994 study showed that fetal hepatocytes injected intraperitoneally into ten patients with fulminant hepatic failure induced the recovery of some of them, with neurological improvement and lowered ammonia and bilirubin levels recorded,...
with no procedure-related complications [15]. There has been a case report of a patient diagnosed as acute fatty liver of pregnancy. After delivery, the patient progressed to grade IV encephalopathy and did not improve despite intensive clinical management measures. After the infusion of human fetal hepatocytes, she completely recovered [16]. Another case report of a patient with clinical end-stage chronic liver failure, who received two intrasplenic infusions of freshly isolated fetal liver cells, obtained an improved MELD (Model for End-Stage Liver Disease) score, with no signs of encephalopathy within the first 18 months after cell transplantation [38]. Intrasplenic fetal liver cell infusion in patients with end-stage chronic liver disease, who are on the waiting list for liver transplantation, has also been recently reported to induce a positive effect on both clinical scores and encephalopathy [53]. A recent study has suggested that rat fetal hepatocytes may have antifibrotic properties when transplanted into damaged livers, which would have the dual benefit of supporting parenchymal regeneration while targeting the scarring component of chronic liver disease [54]. This may have two unresolved explanations: this finding is a general phenomenon, seen using human fetal hepatocytes, particularly in the chronic liver injury setting; fetal cells could be less immunogenic compared with adult hepatocytes.

3.2 Neonatal Hepatocytes

Livers from neonatal donors are not normally used for transplantation because of their small size and the technical difficulties of performing vascular sutures. However, a high yield of good functional quality hepatocytes can be isolated from neonatal liver tissue [17–19]. Liver tissue is enzymatically dissociated using a two-step collagenase perfusion technique. Major liver vessels are canulated and perfused at 37 °C at a flow rate of 100 ml/min with a calcium-free buffered salt solution that contains 0.5 mM chelating agent EGTA. Then the liver is perfused with a calcium-containing collagenase buffered salt solution for 10 min to digest the extracellular matrix [9, 19]. After digestion, liver tissue is disrupted, and suspended hepatocytes are purified by filtration and low-speed centrifugation [19]. The hepatocyte number and cell viability are assessed by the trypan blue exclusion technique and are usually higher than 90%. The average hepatocyte yield is 15–20 × 10⁶ hepatocytes per gram of liver tissue [19] (Table 2).

Neonatal hepatocytes show similar hepatic functionality to an adult liver. The ureogenic capability of neonatal hepatocytes is comparable to that reported for adult hepatocytes [9, 19] (Table 2). In fact, Meyburg et al. [18] transplanted neonatal hepatocytes from the same donor to four different pediatric patients to treat urea cycle disorders with considerable beneficial therapeutic effects. Regarding the expression of CYP450 enzymes, significant increases in expression to mature levels for most enzymes occur only after birth [45, 46]. Moreover, functional CYP450s in neonatal
hepatocytes display a similar balance to that in the adult liver [19, 55]. A recent study has revealed that CYP3A4 and CYP2C9 give the highest activity values in all the neonatal hepatic cell preparations, followed by CYP2A6, CYP2B6, and CYP2E1 with lower levels, and finally by CYP2C19 and CYP2D6 with the lowest activity values, where CYP1A2 was almost undetectable [19] (Fig. 2). The relatively strong CYP3A4 activity noted in neonatal hepatocytes is a good indication of their metabolic competence because the drugs commonly used in standard immunosuppression for HT (e.g., tacrolimus, cyclosporine, or prednisone) are metabolized by this enzyme. Previous reports have reported similar patterns of human hepatic CYP enzymes in the neonatal liver [45, 56, 57].

Some drug-metabolizing enzymes do not become active until a certain age is reached and, conversely, others appear to be higher compared with adults.

Glucuronidation activity in neonates has been described to develop from minimal to almost adult levels within months of birth and has shown extensive interindividual variability [46, 48]. Particularly, the expression of UGT1A1 in humans is modulated in a developmental fashion and reaches adult levels by 14 weeks of postnatal life [46, 48]. In the study of Tolosa et al. [19], the expression of UGT1A1 and UGT1A9 was detected in all the hepatocyte preparations, while enzymatic activity was measured only in some preparations, probably due to lack of maturity of the enzymes [46, 48]. However, isoforms UGT1A6 and UGT2B7 showed considerable activity in all the neonatal preparations, thus providing them with a detoxification capability by the conjugation and elimination of hydrophobic compounds [19].
Postnatal human livers as fetal livers have been shown to contain pluripotent hepatic progenitors, which are EpCAM+ [43, 58, 59]. If compared to the adult liver, the hepatocyte isolation procedure in neonatal livers provides cell suspensions with a higher proportion of hepatic progenitor cells (EpCAM+ staining), which can also participate in the regeneration of liver parenchyma after transplantation (Table 2). These results could imply a key advantage, particularly for neonatal hepatocytes used as a source of high quality cells to improve hepatic cell therapy applicability. Another major issue for neonatal human hepatocytes is that they are diploid [60] and can pass through the usual mitotic cycles several times, and they maintain their initial ploidy, while the liver cells polyploidization process is generally considered to indicate terminal differentiation and senescence leading to diminished replication capacity [61]. Therefore, the larger number of progenitor cells and diploidy maintenance through several mitotic cycles confer excellent advantages to the hepatic cells isolated from early neonatal livers over adult ones to help improve engraftment, proliferation, and long-term survival in the host liver.

Neonatal liver cells seem less vulnerable to cryopreservation effects than adult hepatocytes and can be stored in liquid nitrogen with no significant loss of viability and function postthawing [6, 19]. Apoptosis has been identified as an important cause of freshly isolated and banked hepatocyte death, with mitochondria as the key players in the initiation of apoptosis and in cryopreservation-induced cell damage [62]. Neonatal hepatocytes present smaller apoptotic and necrotic cell numbers after thawing if compared with adult ones [19] (Table 2). These findings are quite likely related to the fact that the mitochondrial function is not substantially altered by cryopreservation in neonatal cells [17]. In addition, a higher expression of adhesion molecules (β1-integrin, β-catenin, and
e-cadherin), and better attachment efficiency and cell survival have been reported after thawing [19]. One major problem commonly found after HT is the small number of transplanted cells found in the graft. Both the membrane integrity and adhesion molecules involved in cell–cell and cell–extracellular matrix adhesion mechanisms need to be preserved for engraftment into the host liver to be a success [63]. In agreement with these facts, there are reports which indicate that transplanted newborn [64] and fetal [65] rat hepatocytes, and neonatal mouse hepatocytes [66], can integrate, expand, and differentiate by producing more colonies in the host liver than adult liver cells, irrespectively of the recipient’s age. This suggests an improved engraftment and survival advantage of transplanted neonatal hepatocytes in the host liver as opposed to adult hepatocytes. In summary, high functional hepatocytes can be particularly obtained from neonatal livers, which can extend the pool of available organs for cell transplantation.

**3.3 Embryonic Stem Cells**

PSCs may offer many advantages as an alternative cell source for transplantation because of their availability and ability to be expanded in vitro or in vivo. Stem cells may have the potential to produce a more sustained significant metabolic correction but must be shown to be effective in controlled trials. Stem cells for cellular therapy of liver disease have been extensively reviewed [3, 21, 26, 67–69].

ESCs are self-renewing PSCs that can be isolated from the inner mass of a blastocyst. Since the source of ESCs needs the blastocyst to be destroyed, some ethical concerns have arisen. These cells proliferate extensively in vitro, differentiate into derivatives of all three germ layers, and express a number of characteristic markers (Oct4, SSEA-4, TRA-1-60, and TRA-1-81) with high levels of telomerase activity [70]. Studies on liver development have identified crucial signals for the hepatic lineage, which include Activin A, FGF, BMP, HGF, Oncostatin M, and dexamethasone [4, 71, 72]. From all this, different protocols have been developed to differentiate ESCs into HLCs. The obtained phenotype seems to come closer to fetal hepatocytes than adult ones [73] although in vivo, these cells could mature and achieve a more similar phenotype to adult cells. In this sense, different attempts, which include their culture in 3D systems [74], have been recently described.

The potential to differentiate human ESCs in vitro and to provide an unlimited source of hepatocytes for use in biochemical research and the treatment of liver disease are most promising [75]. Although animal models have provided encouraging results [21], the clinical application of ESCs has always been associated with practical and ethical concerns. Therapeutically useful differentiated ESCs must be safe (i.e., nontumorigenic) and need to contribute to liver function in vivo. However, the potential of ESCs and their differentiated progeny to generate spontaneous tumors is
of particular concern for clinical applications. Initial studies have shown that ESCs injected in their undifferentiated state into mice resulted in teratoma formation, which killed the animals [76], while no teratoma was produced when differentiated cells were injected. Yet some reports have indicated tumor formation after the transplantation of ESC-derived hepatic cells despite predifferentiation [77, 78], and transplanted cells containing a number of undifferentiated ESCs [79]. Other reports have shown that transplantation of highly differentiated cells does not entail the development of tumors [80], thus suggesting that steering ESCs to an appropriate state could be an important step for safe and effective cell therapies. To this end, well-defined methods to reduce the tumorigenicity of transplanted cells, including protocols for complete terminal differentiation and the very strict elimination of undifferentiated ESCs from transplanted cells, should be established [81, 82].

iPSCs are human somatic cells that have been reprogrammed to a pluripotent state. They are the equivalent to ESCs in gene expression, but can be reliably derived from adult tissue and have been shown to differentiate efficiently into HLCs [23, 83]. Although many protocols have been defined to differentiate iPSCs, they still differ from adult hepatocytes [84], and different strategies such as their culture in a 3D system [85] have been described to increase their maturity. On top of this, the latest advances made in gene editing technologies have vigorously endorsed the possibility of obtaining disease-free autologous cells from patient-specific iPSCs (Fig. 3). Fascinating progress has been made with the demonstration of the ability of human iPSCs to form small liver organoids with metabolic function when cocultured with endothelial and mesenchymal cells [86].

Human iPSCs provide a unique opportunity to generate live cellular models of patient-specific diseases for personalized cell therapy and for screening candidate pharmacological molecules (Fig. 3) [87]. For monogenic metabolic disorders, the use of iPSCs would enable the study of the effect of mutations on the differentiation and/or function of cells. The liver engraftment potential and regenerative capabilities of human iPSC-derived hepatic cells in vivo have been recently demonstrated [88].

Personalized cell therapy using iPSCs would avoid rejection and, thus, immunosuppression. However, there is still some controversy about the immunogenicity of these cells, which should be assessed before these autologous cells are clinically used [89]. On top of this, such a therapeutic approach would require correcting the genetic anomalies that induce the disease. In this sense, several strategies, such as the use of lentivirus, Zinc Finger Nucleases, or transcription activator-like effector nucleases, have been assessed [87] (Fig. 3). Targeted gene correction of pathological mutations
in patient iPSCs is promising for both regenerative medicine and in vitro disease modeling. The differentiation derivatives from corrected patient iPSCs have the potential to be employed in autologous cell therapy. However, it is essential to ensure that gene editing procedures do not introduce any unexpected mutation. To this end, more efficient and safer gene correction strategies, as well as more robust whole-genome sequencing tools, may be needed to generate mutation-free iPSCs before being therapeutically applied in the future.

3.5 Direct Reprogrammed Cells

The development of protocols that differentiate PSCs into HLCs has been limited by the need for information about the mechanisms implicated in the functional maturation of the human liver after birth [90]. If cells are directly reprogrammed, this limitation may be evaded. Moreover, this strategy would be presumably less tumorigenic, provided that integration-free gene delivery methods are used [87]. Different publications have demonstrated the production of highly functional human HLCs using direct
reprogramming approaches [91, 92]. Yet their use for cell-based therapies remains unclear because the currently available technologies to generate them and to increase their proliferative ability are incompatible with in vivo use [90].

Liver damage or loss of liver mass can extensively stimulate regenerative capacity until the hepatic mass has been restored by the proliferation of mature parenchymal liver cells [93, 94]. However when liver regeneration is impaired, hepatic progenitor cells (HPCs), with the bipotential capability of generating both biliary epithelia and hepatocytes, become active and replace damaged cells [95]. Organoids have also been derived from human livers by the EpCAM selection of bipotential hepatic epithelial cells [96]. Recently, different studies in animals have suggested that hepatocytes supply all the parenchyma’s regenerative capacity [4, 97, 98], although other recent studies in mice have shown the importance of HPCs in liver injury recovery [4, 99]. A key issue would be to determine if studies in rodents can be extrapolated to human disease.

HPCs isolated from human fetal liver are less immunogenic, highly propagative, and more challenging for cryopreservation than adult ones [100]. Fetal liver stem cells have been identified as a transition between embryonic cells and adult ones, which is mostly nonteratogenic [101]. During the third trimester of fetal development, plasticity to form liver parenchyma cells renders fetal HSCs an excellent resource for cellular therapeutic approaches [100].

Mesenchymal stromal cells (MSCs), formerly known as mesenchymal stem cells, are multipotent nonhematopoietic adult stem cells that have been isolated from a variety of tissues, including bone marrow and adipose tissue [102, 103]. They can readily differentiate into a variety of cell types, including osteoblasts, chondrocytes, adipocytes, and hepatocytes [104]. In the last decade, several studies have reported the plasticity of MSCs toward a functional hepatocyte-like phenotype, which suggests the potential for clinical applications. Based on knowledge about the onset of hepatogenesis and liver development [104], different strategies have been attempted to induce hepatogenic trans-differentiation of MSCs into functional HLCs. They are based on either the use of a cocktail of exogenous factors or multistep trans-differentiation protocols by sequential exposure to several factors, which reflect their temporal expression during in vivo hepatogenesis [102, 105–108]. Preclinical and clinical studies have suggested that MSC transplantation can moderately restore liver function in liver diseases [105, 109, 110]. Yet the benefits of MSCs have been suggested to be related more with the expression of immunomodulatory factors than engraftment and subsequent hepatic function of the transplanted cells [111]. In fact, MSCs have been reported to be a therapeutic option for inflammatory conditions. Moreover, the efficacy of MSCs, when cocultured with hepatocytes, provides combined hepatic and anti-inflammatory therapy in ALF [110].

3.6 Other Potential Cell Sources
Human amnion epithelial cells (hAECs) isolated from term placenta are an important cell source for generating HLCs. Cells isolated from the placenta have been the subject of intense research because many of these cells express characteristics of multipotent or even PSCs. hAECs are abundant, have similar surface markers and gene expression profiles to those reported for human ESCs and MSCs, respectively, and differentiate into lineages of the three embryonic germ layers, including the HLCs that originate from the endoderm [112, 113]. Under defined culture conditions, hAECs can adopt hepatic characteristics [113–115]. Cells isolated from amnion also have some unique properties compared to some other stem cell sources, in that they are isolated from a tissue that is normally discarded following birth, they are quite plentiful and easily isolated, and they do not produce tumors when transplanted. Cells isolated from the amnion may be a uniquely useful and non-controversial stem cell source [113, 116]. Preclinical studies in animal models have shown that hAECs are able to correct the characteristic biochemical imbalances of liver diseases with minimal manipulation, which would encourage the isolation and banking of these cells to be used in the clinical practice for transplantations of patients with liver disease [116].

As a general consideration, the safety, engraftment, and functionality of HLCs are key issues that will need more work before the full potential of using stem cells to treat end-stage liver failure can be achieved.

4 Cell Cryopreservation

Cryopreservation and banking allow cells to be stored for a long period of time until they are required for both scheduling and emergency treatments, which thus improves clinical outcomes. Cryopreserved cells have some advantages in that they are constantly available, and extensive quality testing can be performed to determine suitability for transplantation, and to customize cell preparations for each receptor and sterility testing [9, 117, 118]. On the one hand, cryopreservation is accomplished by controlling the freezing rate in a solution that contains a permeable cryoprotectant, usually DMSO, and involves a thawing method. On the other hand, it is known that current cryopreservation and thawing procedures cause detrimental effects on the viability and functionality of adult human hepatocytes. Thus upon thawing, they are often unsuitable for clinical use [6, 9–11]. Better cell storage is, therefore, a priority, and refinements of freezing protocols to better preserve hepatocyte functionality in order to improve the performance of single cryopreserved cells after thawing have been implemented [6, 9–11]. Alternatively, it has been reported that microencapsulation techniques have the potential to protect
hepatocytes from cryoinjury, which would not only allow the efficient recovery of functional and morphological integrity after thawing, but prevent immune cell-mediated rejection upon transplantation into allogeneic recipients [119, 120]. Cryopreservation methods of microencapsulated cells with variable effectiveness have been reported. Recently, optimized protocols to produce alginate-microencapsulated adult human hepatocytes suitable for clinical transplantation, which could be adapted to encapsulate fetal, neonatal hepatocytes or stem cell sources, have been established [121, 122]. Indeed, intraperitoneal transplantation of microencapsulated hepatocytes in animal ALF models has provided promising outcomes, which indicates that this strategy should be suitable for emergency treatments in conditions such as ALF [121, 122].

Similarly, optimized cryopreservation protocols for single cell suspensions of HLCs derived from stem cells are needed for wide clinical use. Thus, successful HLCs cryopreservation to retain wide viability, and their hepatic differentiated status, would allow their banking, which would, in turn, offer the advantage of having cells readily and limitlessly available for clinical purposes. As for hepatocytes, it has been recently reported that functional HLCs derived from hAECs can be microencapsulated within alginate without losing viability or function in vitro, and could likely be banked for clinical use [115].

5 Clinical Trials

Based on the initial and heartening results obtained in liver cell therapy, different programs, sponsored mainly by biotech companies, currently evaluate the safety and efficacy of liver cell transplantation in controlled clinical trials.

The Cytonet program uses cryopreserved cells for intraportal administration. This program consists of a dispersion of cryopreserved liver cells prepared from nontransplantable organs and refined under Good Manufacturing Practice conditions, and their application in patients with urea cycle disorders [123, 124]. In order to determine the effectiveness of this therapy, an in vivo 13C-ureagenesis assay has also been proposed [123, 125]. Furthermore, an extracorporeal liver assist device system has been developed by Vital Therapies for acute-on-chronic liver failure [124]. The Promethera program employs liver-derived progenitor cells that are expanded in vitro and injected into patients with various hereditary metabolic diseases [123, 124]. Although preclinical studies in animals have shown that these cells are able to engraft and proliferate in the recipient liver [126], the clinical outcome of the first transplantation in a 3-year-old girl with OTC deficiency has not yet been reported [127]. The use of EpCAM+ cells to treat different liver diseases has been assessed at the Liver Institute in
Hyderabad in India. A representative early publication has revealed that 25 patients with liver cirrhosis of different etiologies were infused with human fetal liver-derived stem cells (EpCAM+), who obtained improved mean MELD scores [128]. However, details on these patients’ long-term outcomes are still not available, and further information is needed to elucidate the potential qualities and efficacy of these strategies [129].

Currently, more than 20 clinical trials focus on treating liver diseases with MSCs [129–131]. In this sense, Peng et al. [134] showed good short-term efficacy, but long-term outcomes were not marked when autologous bone marrow MSCs were transplanted in patients with liver failure caused by hepatitis B. Although studies of cell-based therapies in cirrhosis are very heterogeneous in terms of the types of infused cells, it has been evidenced that some forms of cell-based therapies may transiently improve liver function in some patients with cirrhosis [132–135]. However, a recent randomized controlled trial that used autologous bone marrow MSCs transplantation offered no beneficial effect in cirrhotic patients [132]. So, although the outlook of using MSCs as cell therapy to treat liver diseases is encouraging, a better understanding of the mechanism that underlies their therapeutic effects, and a better validation in preclinical and clinical settings, are required [131].

The current challenge of these second-generation liver cell transplantation strategies is to develop reliable differentiation protocols that confer sufficient maturity to differentiated cells for widespread clinical use, in addition to the evaluation of the immunogenicity, toxicity, and tumorigenicity of cells [4, 124].

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Experimental Hepatocyte Transplantation
Hepatocyte Transplantation
Methods and Protocols
Stock, P.; Christ, B. (Eds.)
2017, XIII, 365 p. 68 illus., 52 illus. in color., Hardcover
A product of Humana Press